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Sphingomyelin and cholesterol modulate sodium coupled uptakes in proximal tubular cells

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Sphingomyelin and cholesterol modulate sodium coupled uptakes in proximal tubular cells. Sphingomyelin (SM) and cholesterol are major lipid species of apical membranes in renal proximal tubular cells and confer to these membranes a low fluidity. Changes in membrane fluidity and/or lipidic composition were shown to affect the activity of cotransport systems of renal apical membranes. We evaluated the effect of decreasing membrane SM content on lipidic composition, membrane fluidity and sodium (Na)coupled uptakes in rabbit proximal tubular cells in primary culture. Sphingomyelinase (SMase) (30 to 250 mU/ml) decreased [^{3}H]choline-labeled SM content, decreased cholesterol content, and increased cholesterol esterification. SMase did not modify membrane fluidity on isolated brush border membranes. SMase decreased V_{max} of Na-dependent uptake of phosphate and α-methyl-D-glucoside, but not of alanine. SMase did not influence protein kinase C-induced inhibition of phosphate and glucose uptake. Increasing membrane cholesterol content with cholesterol-enriched liposomes subsequently to SMase action restored in part glucose uptake, but not phosphate uptake. In conclusion, SM degradation affected Na-phosphate and Na-glucose cotransports through changes in both SM and cholesterol contents of apical proximal membranes; these changes seemed to occur independently from changes in bulk membrane fluidity. These results suggest that SM and cholesterol have distinct and intricated roles in accessibility and/or activity of apical cotransport systems.

Apical membranes of renal proximal tubulal cells (PTC) are remarkable by their low fluidity, related to the abundance of sphingomyelin (SM) and cholesterol in their constituent lipids [1, 2; reviewed in 3, 4]. The outer leaflet of apical membranes is markedly richer in SM than the cytoplasmic counterpart: SM accounts for more than 75% of phospholipids in the outer leaflet [5]. Maintenance of the asymmetry in lipidic composition and fluidity between the apical and the basolateral domains of the plasma membrane, on the one hand, and between the external and the cytoplasmic leaflets of the apical membrane, on the other hand, relies in part upon the integrity of tight junctions [3, 4].

The activity of transport proteins located in apical, brush border membranes (BBM) is modulated by changes in the physical state and/or composition of membrane lipids [4]. Increasing fluidity of apical membranes either with the local anesthetic drug, benzyl alcohol, or by opening of tight junctions increased Na-phosphate (Pi) cotransport [6–8], decreased Na-glucose cotransport [6, 7, 9], but did not affect Na-alanine cotransport [7, 10]. Cholesterol content appears to be an important modulator of the activity of the Na-Pi cotransport system as indicated by two lines of evidence: (i) during a low-Pi diet, increased tubular Pi reabsorption was associated with a decreased cholesterol content of renal BBMs [2, 11] and, after subsequent enrichment of BBMs with exogenous cholesterol, Pi uptake by BBM vesicles returned to its control value [11]; (ii) BBM vesicles of aging rats are characterized by a lower Na-dependent Pi uptake and a higher cholesterol content than those of young animals [12].

The possibility that SM content might influence renal transport was raised from results of in vivo experiments: first, renal ischemia induced a dramatic decrease of Na-coupled glucose uptake and SM content in renal BBMs [13]; second, treatment of rats with gentamicin decreased both Pi uptake and SM content in BBM vesicles [14]. Accurate interpretation of these effects, however, is made complex by recent evidence for reciprocal influences of SM and cholesterol on their respective contents in plasma membranes [15, 16].

The aim of the present study was to evaluate, in renal PTC grown in primary culture, the effect of a decrease of membrane SM content, induced by SMase, on Na-dependent uptake of Pi, glucose, and alanine, as well as on lipid composition and membrane fluidity. We show that SMase depressed Na-Pi and Na-glucose cotransport, and that this effect is related to decreases of SM and cholesterol content, but not to changes in bulk membrane fluidity.

Methods

Materials

Ethanolamine, cholesterol, egg phosphatidylcholine (PC), insulin, transferrin, dexamethasone, triiodothyronine, α-methyl-D-glucopyranoside (MGP), L-alanine, Na selenite, type I collagenase, phorbol 12-myristate 13-acetate (PMA), bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Percoll was purchased from Pharmacia AB (Uppsala, Sweden). SMase (*B. Cereus*) was from Boehringer Mannheim (Germany) and R 59022 from Janssen Life Science products (Olen, Belgium). 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes Inc. (Junction City, Oregon, USA). Lipid suspensions (small unilamellar

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vesicles) were prepared by sonication from cholesterol and egg PC as described [17]. Calculated cholesterol/PC ratios were 0.8 or 2. At the end of preparation, actual ratios were measured and were found to be 0.8 and 1.7, respectively. Liposomes were diluted in culture medium in order to obtain final cholesterol concentrations of 250 μg/ml and 625 μg/ml for liposomes with a cholesterol/PC ratio equal to 0.8 and 2, respectively. By this procedure, PC concentration was the same in the two liposome suspensions. Tracers were from the following sources: $K_2H^{32}PO_4$ from New England Nuclear (Boston, Massachusetts, USA), methyl-α-D-[U-^{14}C]gluco-pyranoside [^{14}C]MGP, [9,10(n)-^{3}H]palmitic acid, [methyl-^{14}C]choline chloride, and L-[2,3-^{3}H]alanine from Amersham (Amersham, UK). Culture media and reagents were from Gibco-BRL (Cergy-Pontoise, France). Plasticware was from Costar (Cambridge, Massachusetts, USA). Silica gel thin-layer plates used for thin-layer chromatography (TLC) were from Whatman (Clifton, New Jersey, USA). All other reagents were of analytical grade.

Cell culture

Primary cultures of renal PTC were prepared as described previously [7, 10]. Briefly, kidneys were removed aseptically from anesthetized New Zealand rabbits (0.9 to 1 kg), decapsulated and sliced in 1 mm-thick sections which were kept at 4°C in Hank's balanced salt solution supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) and 5 mM D-glucose, pH = 7.4 (HBS-Hepes). Cortex was separated from medulla, cortical slices were rinsed three times in HBS-Hepes, and placed in a mixture of 5 ml HBS-Hepes, 5 ml of culture medium, 0.25 ml of 10% bovine serum albumin, and collagenase (final concentration = 0.75 mg/ml). This suspension was transferred to a trypsinizing flask, and was incubated under gentle stirring, during 50 to 60 minutes, at 37°C in a 5% CO_2-95% air atmosphere. After that period, the mixture of renal tubules was washed in HBS-Hepes and centrifuged (200 × g; 3 min; 4°C). The pellet was resuspended in the same solution and the operation was repeated three times. The pellet was suspended in 5% albumin HBS-Hepes solution, kept in ice for five minutes, and centrifuged as above. Homogeneous populations of nephron segments were separated by Percoll centrifugation. The mixture of tubules was suspended in 50% Percoll made isotonic with 10× concentrated HBS-Hepes, and was centrifuged (17,000 rpm; 30 min; 4°C) in a Kontron centrifuge equipped with a A8.24 rotor ensuring 14,500 to 27,000 × g. The F_4 layer, made of proximal tubules, was removed, suspended in HBS-Hepes, and was washed and centrifuged (200 × g; 2 min; 4°C) three times in this solution. The final pellet was suspended in culture medium and tubules were seeded in 6-well (≈10^5 fragments/well) or 24-well plastic trays (2 to 5 × 10^4 fragments/well) which had been coated with NH_3-reconstituted rat's tail collagen. Serum-free culture medium consisted in a 1:1 (vol/vol) mixture of Ham's F-12 and Dulbecco's modified Eagle's medium containing 25 mM Hepes, 21.5 mM HCO_3, 1 mM Na pyruvate, 10 ml/liter of a 100× non-essential amino acid mixture, 4 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 nM Na selenite, 5 μg/ml insulin, 35 μg/ml transferrin, 20 μM ethanolamine, 5 nM triiodothyronine, and 50 nM dexamethasone. Medium was changed after three days, and then on alternate days. Monolayers of proximal cells reached confluence after five to six days, and they were used for experiments two or three days after confluence was achieved. On the day prior to experiments, culture medium was changed to hormone-free medium.

Lipid analysis

For determination of [^{14}C]choline-labeled SM content, confluent cells grown in 6-well trays were incubated in hormone-free culture medium for 48 hours with [^{14}C]choline (1 μCi/ml). At the end of this period, cells were washed three times with culture medium and then incubated during one or two hours in the absence or presence of SMase, in the presence of 5 mM unlabeled choline prior to lipid extraction. The effect of SMase on cholesterol esterification was determined by incubating cells with [^{3}H]palmitic acid (0.5 μCi/ml, 1 hr) prior to addition of SMase. Lipids were extracted according to Hara and Radin [18] with a hexane/isopropanol mixture (3:2, vol/vol) and sonication of the samples. Extracts were evaporated to dryness under a nitrogen stream, and solubilized in a chloroform/methanol mixture (2:1, vol/vol). Neutral lipids were separated on K5 silica gel plates using a hexane/diethylether/acetic acid mixture (130:30:1.5, vol/vol) as developing solvent. Phospholipids were separated on LK5 plates with chloroform/ethanol/water/triethylamine (35:30:6:35, vol/vol) as developing solvent [19]. Individual components were detected by exposure to a primuline spray and visualized in UV light, and identified by comparison with authentic standards [19]. The spots were scraped off, transferred to scintillation vials, and counted by liquid scintillation. For quantification of cholesterol content, cholesterol was eluted from silica gel with chloroform after scraping and its concentration was determined enzymatically [20]. Known amounts of cholesterol and defined phospholipids were treated in the same way to be used as internal standards.

Measurement of membrane fluidity

Rabbit BBMs were prepared according to Booth and Kenny [21]. BBMs were incubated in a buffered saline solution (150 mM NaCl, 20 mM Hepes, pH = 7.4), at 37°C, in the absence or presence of SMase (1 U/ml) up to 60 minutes. At the end of incubation, reaction was stopped with addition of EDTA (final concentration: 20 mM). BBM were washed and centrifuged (16,000 rpm, 20 min) twice in the EDTA solution, and finally resuspended in buffered saline for a 90-minute incubation at 37°C with DPH (final concentration: 1 μM). Fluorescence polarization measurements were performed on a SLM 4800 apparatus (SLM Inc., Urbana, Illinois, USA) as previously described [1]. Light scattering was reduced to less than 1% by the use of cutoff filters. In all conditions, the individual values obtained were the means of at least four successive measurements that by themselves were the average of 10 determinations. Results of steady state depolarization experiments, which reflect the order of membrane lipids, that is, the static component of membrane fluidity, were expressed in terms of fluorescence anisotropy, r, with $r = I_{\parallel} - I_{\perp} G/I_{\parallel} + 2I_{\perp} G$ where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed with the analyzing polarizer parallel and perpendicular to the polarized excitation beam. A correction factor, G, equal to $I'_{\parallel}/I'_{\perp}$, the primes indicating excitation polarized in a perpendicular direction, was used to correct for the unequal transmission of differently polarized light [1].

Uptake studies

Uptakes of Pi, MGP, a non-metabolized analog of D-glucose which is uptaken through the Na-glucose cotransporter, and alanine (Ala) were performed as previously described [6, 7]. Briefly, uptakes were performed at 37°C in a buffered solution with the following composition (mmol/liter): 137 NaCl/5.4 KCl/1 $CaCl_2$/1.2 $MgSO_4$/15 Hepes (pH = 7.4). The sodium-free solution was made by replacing sodium chloride with choline chloride. After removal of culture medium, cells were washed with 1 ml/well of the uptake solution, and were incubated for various periods of time in the presence of $K_2H^{32}PO_4$ (0.5 μCi/ml) or [^{14}C]MGP (0.5 μCi/ml) or L-[^{3}H]alanine (1 μCi/ml), and appropriate concentrations of KH_2PO_4, MGP, or L-alanine. We have previously reported that, for the chosen time of incubation, uptakes increased linearly with time [10]. All these steps were performed at 37°C. At the end of incubation, the uptake was stopped by washing the cells three times with 1 ml/well of ice-cold solution (137 mM NaCl/15 mM Hepes, pH = 7.4). Cells were then solubilized in 0.5% Triton X-100 (250 μl/well) and aliquots were counted by liquid scintillation.

Presentation of data

Cellular content in SM was expressed as the [^{14}C]choline-labeled SM/PC ratio. [^{3}H]cholesterol palmitate content was expressed as dpm/culture well. Uptakes of Pi, MGP, and Ala were expressed as nmol/mg protein [22]. Na-dependent uptakes were calculated by subtracting uptake values measured in the presence of choline from those measured in the presence of Na. Results are presented as mean ± SE of three to five different experiments (N) in which duplicates were obtained. One-way or two-way analyses of variance were performed and, when allowed by the F value, results were compared by the modified t-test [23].

Results

Effect of SMase on SM and cholesterol content, and on membrane fluidity

Incubation of PTC with SMase induced a time- and concentration-dependent decrease of SM content, which reached 52.4% of the control value after a one hour incubation in the presence of 250 mU/ml of the enzyme (Fig. 1A). PC content was not affected by SMase (not shown). In contrast with its effect on SM content, SMase did not modify fluorescence anisotropy of isolated brush border membranes (Fig. 1B): after one hour of incubation in the presence of 1 mU/ml SMase, fluorescence anisotropy of treated BBMs was not significantly different from that of control ones.

Because SMase was reported to affect distribution of cell cholesterol in a kidney cell line [16], we evaluated the effect of the enzyme on cholesterol content and esterification in PTC. After two hours in the presence of 250 mU/ml SMase, cholesterol content was decreased by 18 ± 2.8% ($P < 0.05$; Fig. 2A). In the absence of SMase, [^{3}H]cholesterol palmitate (756 ± 173 cpm/well) represented approximately 3% of cell-associated radioactivity. [^{3}H]cholesterol palmitate content increased under the influence of SMase in a concentration-dependent manner (Fig. 2B), such as the reached value was equal to 3-fold the control one.

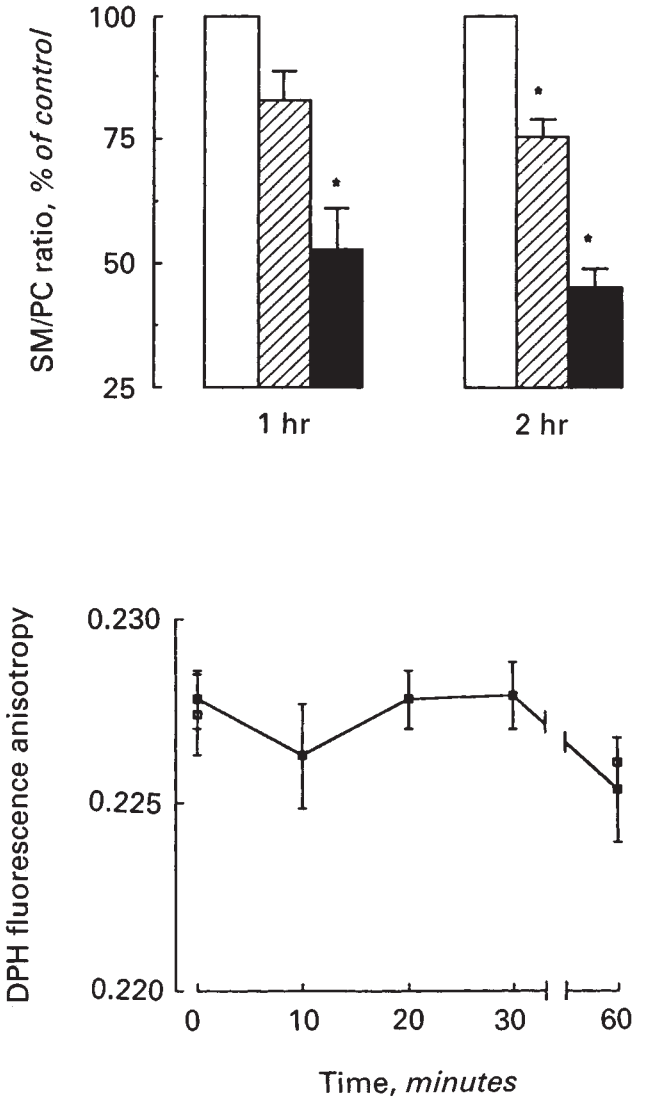

Fig. 1. *Effect of sphingomyelinase on sphingomyelin content and membrane fluidity.* **A.** PTC were incubated with [^{14}C]choline (1 μCi/ml; 48 hr) prior to incubation in the absence (□) or in the presence of 32 mU/ml (▨) or 250 mU/ml (■) sphingomyelinase during the indicated periods of time. Results are expressed as [^{14}C]choline-labeled sphingomyelin (SM)/[^{14}C]choline-labeled phosphatidylcholine (PC) ratio. Data represent means ± SE of three different experiments (N = 3) in which duplicates were obtained. Significantly different from control value, $P < 0.05$. **B.** BBMs were incubated in the absence (□) or in the presence (■) of 1 U/ml sphingomyelinase (SMase) prior to measurement of DPH fluorescence polarization. Results are expressed as fluorescence anisotropy. Data represent means ± SE of three different experiments (N = 3) in which duplicates were obtained.

Effect of SMase on Na-dependent uptakes

SMase decreased Na-dependent Pi and MGP uptakes in a time- and concentration-dependent manner (Fig. 3). In contrast, SMase had no effect on Na-dependent Ala uptake. These changes induced by SMase on uptakes were further characterized by studying the effect of the enzyme on the kinetic parameters of the three transport systems (Fig. 4, Table 1). SMase decreased V_{max} value of Pi uptake from 45.8 ± 5.1 to 26.4 ± 3.0 nmol/mg protein/10 min (Fig. 4A) and decreased

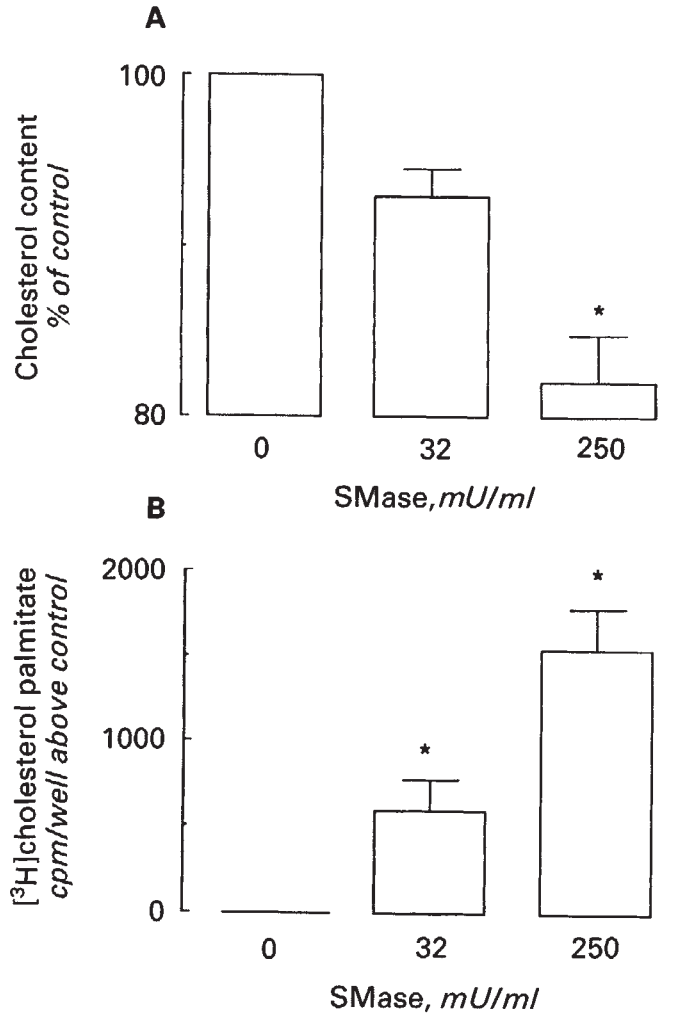

Fig. 2. *Effect of sphingomyelinase on cholesterol content and esterification.* **A.** Cells were incubated during 2 hours in the presence of the indicated concentrations of sphingomyelinase (SMase) prior to extraction and dosage of total cholesterol content. Results are expressed as per cent of control value. **B.** Cells were incubated with [^{3}H]palmitic acid (0.5 μCi/ml, 1 hr) prior to incubation (2 hr) with SMase. Results are expressed as cpm/well above control value (756 ± 173 cpm/well). Data represent means ± SE of three different experiments (N = 3) in which duplicates were obtained. *Significantly different from control value, $P < 0.05$.

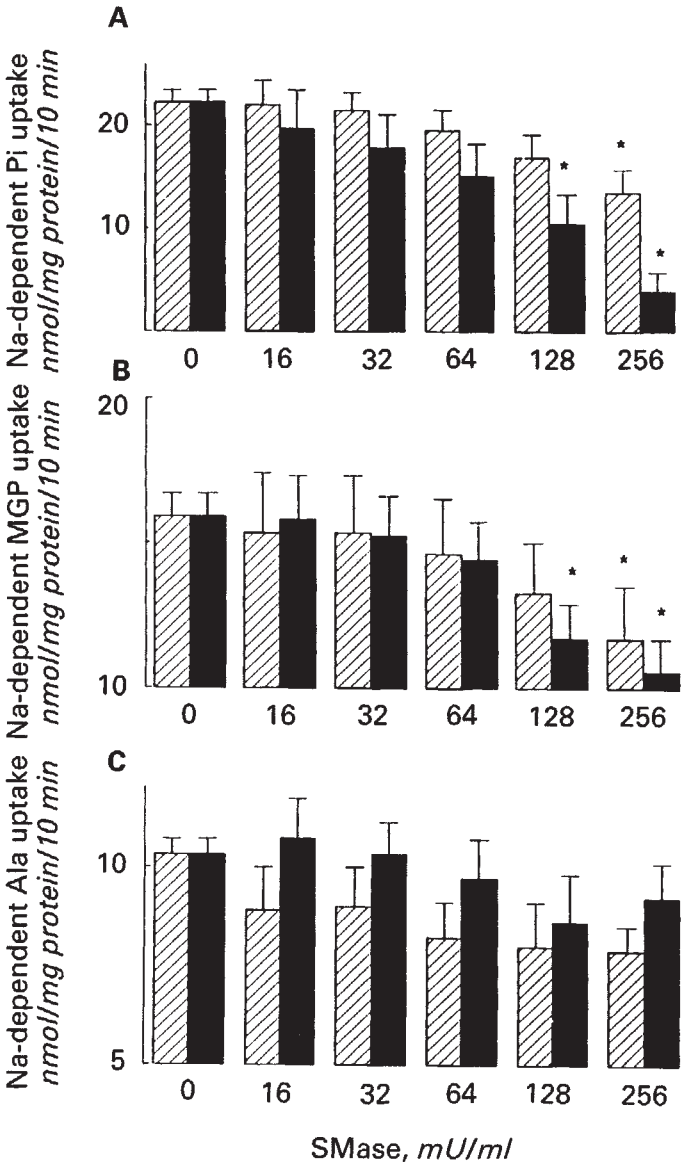

Fig. 3. *Effect of sphingomyelinase on Na-dependent uptake of Pi, MGP, and Ala.* Cells were incubated with sphingomyelinase (SMase) at increasing concentrations for 1 hour (▨) or 2 hours (■) prior to uptake of Pi (0.1 mM, 10 min), MGP (1 mM, 10 min), or Ala (1 mM, 5 min). Data represent means ± SE of four or five different experiments (N = 4 or 5) in which duplicates were obtained. *Significantly different from homologous control value, $P < 0.05$.

V_{max} value of MGP uptake from 57.2 ± 3.2 to 31.7 ± 1.5 nmol/mg protein/10 min (Fig. 4B), while apparent K_m values were unchanged (Table 1). As regards Ala uptake (Fig. 4), neither V_{max} nor K_m values were modified (Table 1).

We have previously reported that, in PTC, activators of protein kinase C (PKC) such as the phorbol ester PMA or the diacylglycerol kinase inhibitor R 59022 [24] inhibited Na-dependent Pi and MGP uptake, but not Ala uptake [10, 25]. Because SM degradation by SMase leads to formation of sphingoid bases such as sphingosine, a well-known natural inhibitor of PKC [26], we wondered whether SMase could modulate the effect of PKC activators on transport in cultured PTC. For that purpose, we first documented that exogenous PKC inhibitors were indeed able to reverse the effect of PMA on Pi and MGP uptakes. Sphingosine (Fig. 5A), at 100 μM, and staurosporin (Fig. 5, lower panel), at 1 μM, blunted significantly the inhibitory effect of PMA on Pi and MGP uptake. This effect, however, was not reproduced by SMase. As shown in Figure 6, the inhibitory effect of SMase on Pi (Fig. 6A) and MGP (Fig. 6B) uptake persisted to a similar extent whether or not cells were treated with PMA or R 59022.

Finally, we attempted to evaluate the respective roles of the decreased SM content and the decreased cholesterol content in the observed changes in transport. This was achieved by incubating PTC in the presence of liposomes with various cholesterol/PC ratios after cells had been prior incubated with or without SMase. In separate experiments, we verified that

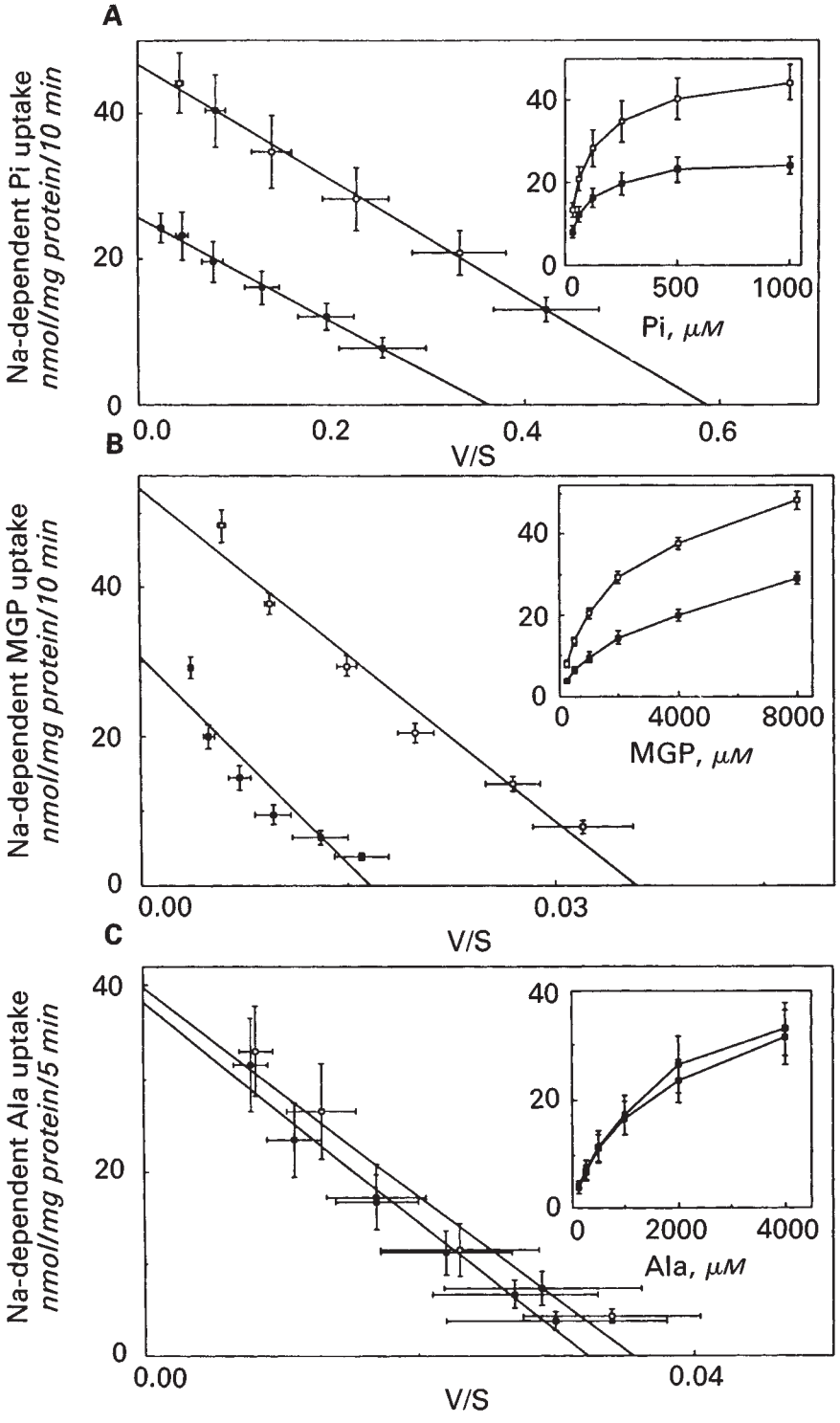

Fig. 4. *Effect of sphingomyelinase on the kinetic parameters of Na-dependent Pi, MPG, and Ala uptake.* Cells were incubated in the absence (□) or in the presence (■) of sphingomyelinase (SMase: 250 mU/ml, 2 hr) prior to uptake of Pi (**A**), MGP (**B**), or Ala (**C**). Kinetic parameters, calculated from Eadie-Hoffstee plots, are given in Table 1.

treatment of cells with liposomes affected cholesterol content of PTC. Indeed, while SMase decreased cellular cholesterol/PC ratio by 18 ± 2.8% ($P < 0.05$), subsequent treatment of cells with cholesterol-rich liposomes restored cellular cholesterol content since cholesterol/PC ratio was only 2.2 ± 1.2% lower than that of control cells (not significant). As shown in Table 2, liposomes had no effect *per se* on uptakes. Liposomes decreased slightly the effect of SMase on Pi uptake, but this effect did not reach significance. In contrast, liposomes reversed significantly the inhibitory effect of SMase on MGP uptake from 60% to 31%.

Discussion

In the present study we have evidenced that, in PTC, decreasing SM content by SMase affected cholesterol content as well, and resulted in a decrease of Na-dependent Pi and MGP

Table 1. Effect of SMase on kinetic parameters of Na-dependent Pi, MGP, and Ala uptakes

	K_m μM		V_{max} nmol/mg protein/10 min	
	Control	SMase	Control	SMase
Pi uptake	81.6 ± 11.6	80.0 ± 8.0	45.8 ± 5.1	26.4 ± 3.0[a]
MGP uptake	1862 ± 249	2167 ± 300	57.2 ± 3.2	31.7 ± 1.5[a]
Ala uptake	1138 ± 157	1267 ± 309	39.0 ± 7.1	36.8 ± 5.9

Cells were incubated in the absence or in the presence of sphingomyelinase (SMase: 250 mU/ml, 2 hr) prior to uptake of Pi, MGP, or Ala. Kinetic parameters were calculated from Eadie-Hoffstee plots shown in Figure 4. Data represent means ± SE of three different experiments ($N = 3$) in which duplicates were obtained.

[a] Significantly different from homologous value without SMase, $P < 0.05$.

uptake, but not Ala uptake. Finally, potential SMase-induced release of sphingoid bases could not blunt the effect of PKC activation on transport.

Effect of SMase on lipid composition and fluidity

The bulk of cellular SM was shown to be located in plasma membranes of many cell types [27]. In proximal tubular cells, distribution of SM was shown to exhibit a double asymmetry: SM is more abundant in apical than in basolateral membranes on the one hand [28], and, within BBMs, SM is mainly located in the outer leaflet rather than in the cytoplasmic one [5]. The reported low turnover rate of SM [28], together with the accessibility of SM to SMase, accounted for the dramatic decrease of SM content under the influence of the enzyme. These results are consistent with those reported in intact cells including a renal cell line [16], and in those obtained in isolated BBMs [5].

Since abundance of SM in membranes was inversely correlated with membrane fluidity [2, 28], it might therefore appear surprising that decreasing SM content in BBMs did not result in a decrease of fluorescence anisotropy value. Such a decrease would have reflected an increase in membrane fluidity. This apparent discrepancy is very likely accounted for by the fact that, in isolated BBMs in which fluidity was measured, SM degradation product generated by SMase, that is, ceramide, most likely accumulated in the membrane and maintained a low degree of fluidity, in part related to the high saturation index of its fatty acids. This situation offers the unique opportunity to dissociate the influence of SM *per se* and of membrane fluidity on transport processes. It contrasts with two other situations in which decreased SM content was associated with an increase in membrane fluidity: (i) the first one is renal ischemia [13]. In that situation, decreased SM content in BBMs was likely related to lateral diffusion of this phospholipid species towards the basolateral domain of the plasma membrane which followed opening of tight junctions [3]; (ii) the second situation was the consequence of early effects of in vivo gentamicin administration on BBMs, in which decreased SM content was associated to an increased PC content and to a decreased fatty acid saturation index [14]. The explanation offered by the authors for these changes was that gentamicin impaired conversion of PC to SM by inhibiting PC:ceramide phosphocholine transferase which catalyzes this reaction [14].

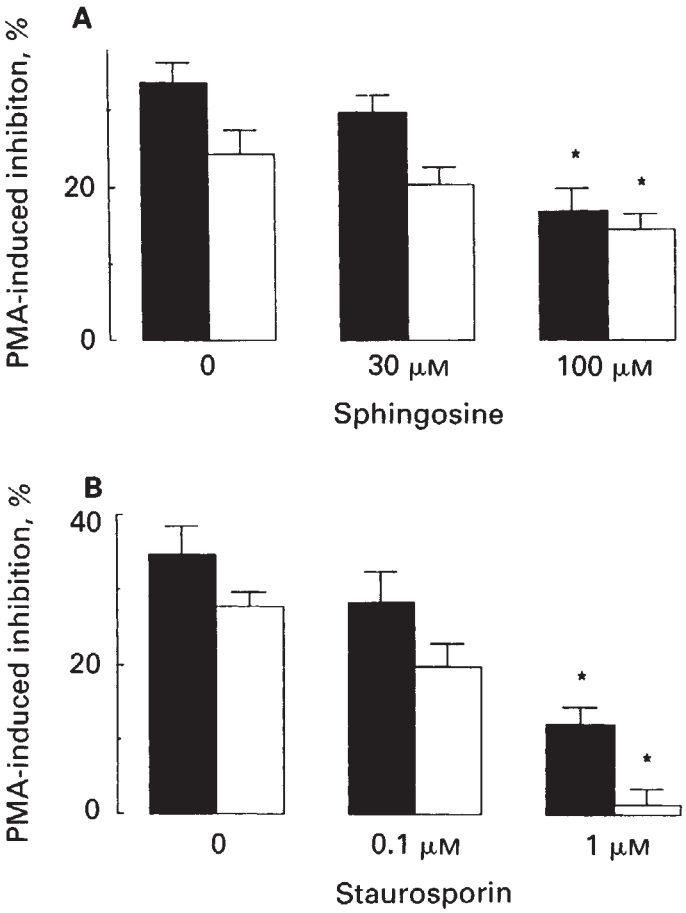

Fig. 5. *Effect of sphingosine and staurosporin on PMA-induced inhibition of Pi and MGP uptake.* Cells were incubated in the absence or presence of PMA (40 nM, 1 hr), with or without sphingosine (**A**) or staurosporin (**B**) prior to uptake of Pi (0.1 mM, 10 min) or MGP (1 mM, 10 min). Results are expressed as PMA-induced inhibition of Pi (■) or MGP (□) (per cent of control values). Data represent means ± SE of four or five different experiments ($N = 4$ to 5) in which duplicates were obtained. *Significantly different from homologous value without SMase, $P < 0.05$.

The effect of SMase in intact PTC was not only a decrease of SM content, but also a decrease of cholesterol content (Figs. 1 and 2). This combined decrease was likely accounted for by colocalization of SM and cholesterol, as evidenced in other systems, and was related to a direct interaction of SM with cholesterol [29]. As a consequence of this interaction, which appears tighter than with other phospholipid species, SM degradation resulted in translocation of cholesterol from the outer leaflet of the plasma leaflet to both the inner leaflet and the intracellular membranes as evidenced by an increase in cholesterol esterification [16]. Our results are therefore in good agreement with previous reports on this phenomenon in other cells [16]. Given this dual effect of SMase, it might be questioned whether the enzyme would have left bulk membrane fluidity unchanged in intact living cells, as this was the case in isolated BBMs (Fig. 1). A positive answer is strongly suggested by the following: (i) evidence was brought that the effect of SM degradation by SMase on monolayer area (which is correlated with fluidity) is the sum of several events: conversion of SM to

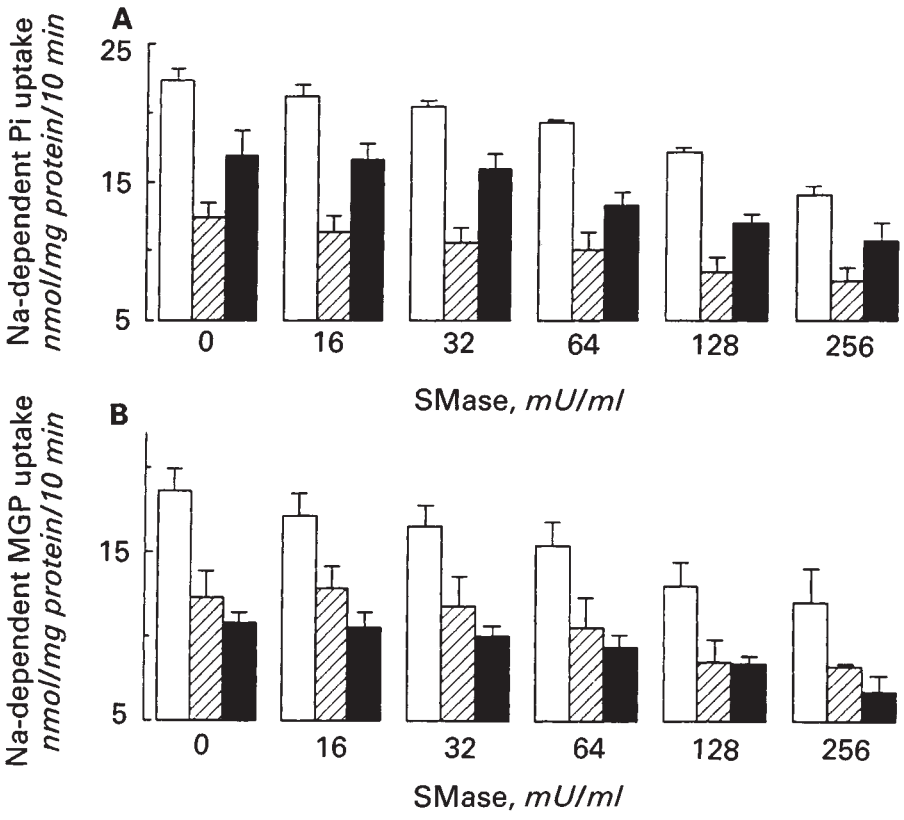

Fig. 6. *Combined effects of sphingomyelinase and PKC activators on Na-dependent Pi and MGP uptake.* Cells were incubated during 2 hours with the indicated concentrations of sphingomyelinase (SMase) prior to Pi (0.1 mM, 10 min) or MGP (1 mM, 10 min) uptake. PMA (40 nM, ▨) or R 59022 (10 μM, ■) were added to the medium during the last hour of incubation with SMase. Data represent means ± SE of four or five different experiments (N = 4 to 5) in which duplicates were obtained. Analysis of variance revealed that the inhibitory effect of PMA or R 59022 persisted in the presence of SMase at any concentration.

Table 2. Effect of liposomes on Na-dependent Pi, MGP, and Ala uptakes

	Control	Liposomes	
		Cholesterol/ PC = 0.8	Cholesterol/ PC = 2.0
Pi uptake *nmol/mg protein/10 min*			
Control	24.3 ± 0.5	24.9 ± 0.8	23.8 ± 0.6
SMase	15.3 ± 0.6[a]	16.3 ± 0.5[a]	17.8 ± 0.8[a]
	(−37 ± 2.0%)[a]	(−34 ± 2.6%)[a]	(−26 ± 2.6%)[a]
MGP uptake *nmol/mg protein/10 min*			
Control	15.1 ± 1.8	14.9 ± 1.9	14.8 ± 2.1
SMase	5.8 ± 0.4[a]	8.7 ± 1.1[a]	10.1 ± 1.2[b]
	(−60 ± 2.7%)[a]	(−40 ± 7.2%)[ab]	(−31 ± 3.1%)[b]
Ala uptake *nmol/mg protein/5 min*			
Control	10.6 ± 0.6	11.0 ± 0.8	11.5 ± 0.9
SMase	9.9 ± 1.1	11.0 ± 1.2	10.9 ± 1.5
	(−7 ± 7.2%)	(−0.6 ± 5.5%)	(−5.9 ± 7.5%)

Cells were first incubated with or without SMase (250 mU/ml, 2 hr). Cells were then rinsed twice with fresh medium and incubated, without SMase, during 2 hr, in the absence or in the presence of liposomes prior to uptake of Pi (0.1 mM), MGP (1 mM), and Ala (1 mM). Data represent means ± SEM of four different experiments (N = 4) in which triplicates were obtained.

[a] Significantly different from the homologous value without SMase, $P < 0.05$

[b] Significantly different from the homologous value without liposomes, $P < 0.05$

ceramide, on the one hand, induced retraction of the monolayer while SMase-induced retrieval of membrane cholesterol, on the other hand, led to expansion of monolayer area. These two opposite phenomena were probably of about equal magnitude in as much as, in cholesterol-containing monolayers, SMase had no significant effect on monolayer area [15]. However, one cannot rule out the possibility that, in intact living cells, ceramide originating from SM degradation was retrieved from the plasma membrane towards intracellular compartments [30], thereby affecting membrane composition and fluidity.

Effect of SMase on transport

SMase-induced decrease of Pi and MGP uptake was not the consequence of changes in Na gradient since SMase was without effect on Na-dependent Ala uptake. This suggests that Na,K-ATPase activity was preserved enough to maintain efficiently a membrane Na gradient. These data are in line with the fact that, in highly polarized epithelial cells like PTC, Na,K-ATPase is located in the basolateral plasma membrane, in which SM amount is markedly lower than in BBMs [28].

Consistent with our observation that SMase decreased Pi uptake is the report that decreased SM content in BBMs of gentamicin-treated rats was associated with a fall of Pi uptake [14]. This decrease of transport occurred despite the concomitant increase in membrane fluidity [14], whereas membrane fluidization was reported to increase the activity of Na-Pi cotransport in both intact renal epithelial cells [6, 7] and in their BBM vesicles [8]. This apparent inconsistency is likely to result from distinct effects of changes in membrane fluidity and composition on the Na-Pi transport system: increase in bulk membrane fluidity increased the V_{max} of Pi uptake [6–8] but did not increase the number of transport units, as estimated from the number of phosphonoformic acid binding sites [8]. This

suggested that increased V_{max} was accounted for by an enhanced activity of an unchanged number of transport units. Similar results (that is, increased Pi uptake and unchanged number of transport units) were obtained when cholesterol content in BBMs was decreased, as during low-Pi diet [11]. In contrast, decreased SM content during gentamicin treatment was associated with a decrease of both the V_{max} of Pi uptake and the number of phosphonoformic acid binding sites [14]. This leads to the conclusion that: (i) SM amount in the outer leaflet of apical membranes plays a key role in the insertion of Pi transport units in the membrane; and (ii) lipidic environment of Pi cotransport units might be SM-rich areas.

Regarding Na-glucose cotransport, there is abundant evidence that membrane fluidization leads to a decrease of its activity [6, 7, 9]. Our results demonstrate that, in addition to changes in fluidity, modifications of lipidic composition may also affect glucose uptake. The influence of SM by itself on the activity of Na-glucose cotransport appears to be limited since glucose uptake was unaffected during gentamicin treatment while SM content was decreased [14]. It is noteworthy that, in this study, cholesterol content was unchanged [14]. On the contrary, combined decreases of SM and cholesterol content, such as those observed in our study with SMase, led to a decrease of glucose uptake (Figs. 3 and 4, Table 1). These data suggest that membrane-bound cholesterol is an important modulator of glucose transport. This possibility was confirmed by the fact that enrichment of membranes in cholesterol with liposomes restored in part MGP uptake. This effect was specific since liposomes were ineffective to modify significantly Pi uptake (Table 2). Moreover, that liposomes had no intrinsic effect on transport in the absence of SMase argues against any toxic effect of the lipid suspension on proximal cells. Renal ischemia was also reported to induce a combined decrease of cholesterol and SM content and a dramatic decrease of glucose uptake by BBM vesicles, while alanine uptake was not affected [13]. Because situations in which a selective decrease of cholesterol content of BBMs, such as administration of a low-Pi diet, were not reported to exhibit a decrease of glucose uptake [2], it may well be that decreases of both SM and cholesterol are mandatory to modulate the activity of this transport.

In GH_3 cells endogenous SMase activity was shown to be stimulated by diacylglycerol, but not by phorbol esters, through a PKC-independent pathway [31]. In turn, SM degradation led to the release of sphingoid bases which exerted a negative feedback control on PKC activity [32]. While the final steps of this feedback loop could not be evidenced in cultured PTC, the effect of increased endogenous diacylglycerol on SM content in these cells is still to be evaluated. Because diacylglycerol content could be increased in PTC [25], it can be speculated that modulation of endogenous SMase activity is of potential interest in modulation of transport in the proximal tubule under physiological or pathophysiological conditions.

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